ALKALOIDS OF TOBACCO

Identification and Determination

The value of some newer techniques in studying the chemistry of tobacco is exemplified by their application to tobacco alkaloids and their derivatives. The newer techniques mentioned are ultraviolet and infrared spectrophotometry, chromatograph, countercurrent distribution, photochemical oxidation, reciprocal grafts with Nicotiana, radioactive tracers, statistical analysis, and improved pyrolysis, distillation, and colorimetric procedures.

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FOR a long time researchers on tobacco chemistry had to be content with studying empirical groups of organic substances —fiber, pectin, protein, alkaloids, amino and amide nitrogen, carbohydrates, polyphenols, and pectins. A wealth of information has resulted, not only on the fresh plant of various types and grades (*36*, *43*, *44*), but on curing, fermentation, and aging (*18*, *19*). It has always been realized, however, that such studies could well be supplemented by determining the individual constituents, if only methods were available for doing so.

Recently chemists have been given many new instruments and procedures for the examination of plant material. Some of these have been applied to all groups of the constituents of tobacco, but it is proposed to limit this discussion largely to the bacco alkaloids. There are at least three reasons for this choice: irst, most of the recent detailed work on tobacco concerns the kaloids; secondly, they are a complicated group and hence well strate what can be done with the newer techniques; thirdly, substances are of vital interest in tobacco processing and in of the physiological effects of smoking.

hemistry of the tobacco alkaloids has been studied for rs. The names of Späth, Wenusch, Pictet, Pinner, and many others bring to mind the important investigations on tobacco carried on without the aid of the newer analytical techniques. Nicotine received the most attention in these earlier works. It was not until Woodward, Eisner, and Haines (*54*) studied the pyrolysis of nicotine that large quantities of homologs became available for study and a new phase in tobacco alkaloids began.

Nicotine is converted to myosmine by pyrolysis over quartz at 570° C. The conversion is about 18% on the basis of the weight of the original nicotine and 33% on the basis of nicotine consumed in the pyrolysis. Myosmine had previously been obtained from tobacco smoke by Späth, Wenusch, and Zajic (*40*). However, its ready availability from the pyrolysis of nicotine afforded the opportunity to study its chemical reactivity.

The study by Haines, Eisner, and Woodward (*27*) showed that the ring structure normally applied to myosmine was easily hydrolyzed in water to the open chain derivative, 3-pyridyl-ω-aminopropyl ketone. The presence of a primary amino group made possible a new and easy determination of myosmine by the Van Slyke method and has been employed most successfully by

Frankenburg (*21*) in his study of the transformation products of nicotine during the fermentation of tobacco.

Various degrees of reduction applied to myosmine have led to numerous other derivatives not readily available from or not yet known to exist in tobacco, such as nornicotine, 3-(4-aminobutyl)-pyridine, and 3-(4-aminobutyl)piperidine.

3,2′-Nicotyrine can now be obtained either as one of the pyrolysis products of nicotine or by the catalytic oxidation of nicotine (*53*). This enabled Haines and Eisner (*26*) to clarify a discrepancy in our knowledge of the tobacco alkaloids. Wibaut and Hackmann (*48*) reduced nicotyrine to dihydronicotyrine and characterized the picrate. Späth, Wibaut, and Kesztler (*41*) hydrolyzed 1-methyl-3-nicotyrinoyl-2-pyrrolidine to *N*-methylmyosmine, and its picrate was identical with the picrate of Wibaut and Hackmann's dihydronicotyrine. Haines and Eisner (*26*) reduced nicotyrine, but the product was not *N*-methylmyosmine. Ultraviolet spectroscopic examination, by Swain *et al.* (*42*), with a chemical test indicated that in dihydronicotyrine the double bond is in the 3′,4′ position, whereas in *N*-methylmyosmine it is in the 2′,3′ position. The recent work of King, Marshall, and Smith (*28*) in redetermining the structure of some supposed 2-phenyl-Δ^3- and -Δ^4-pyrrolines lends support to this contention.

This same work identified the pseudo oxynicotine, made by the method of Pinner and Wolffenstein (*34*) by treatment of nicotine oxide with hydrochloric acid in a sealed tube, to be the open chain 3-pyridyl-3-methylaminopropyl ketone.

Pinner and Wolffenstein also reported obtaining, in addition to pseudo oxynicotine, another isomer, which they termed nicotone. The oxazine could be converted into pseudo oxynicotine by treatment with concentrated hydrochloric acid. Reduction of the oxazine gave the corresponding alcohol, 4-methylamino-1-(3-pyridyl)-1-butanol, which was dehydrated with phosphorus pentoxide to nicotine and metanicotine.

Weil (*46*, *47*) studied the photochemical oxidation of nicotine in the presence of light and methylene blue. The irradiated product appeared at first to be a mixture of at least two derivatives. It has since been shown by paper chromatography and countercurrent distribution techniques at this laboratory that the irradiated product is a mixture of at least six components. Frankenburg (*20*) obtained a product from the fermentation of cigar-leaf tobacco which had a close similarity to the irradiation product. Naghski has proved that nicotine oxide is one of the major constituents of both products (*32*).

Thus, by means of pyrolysis, ultraviolet spectroscopy, paper chromatography, countercurrent distribution technique, and newer chemical procedures, this investigation into the chemistry of nicotine and its homologs has opened the way for their identification in tobacco and its fermentation products, in the metabolites of nicotine in the human body, and for the development of new methods of qualitative and quantitative detection of these alkaloids.

Qualitative Analytical Procedures

Further recent progress in research on tobacco constituents, which has been made possible by new physical tools and new physical methods, is reviewed in the following sections.

Ultraviolet Spectrophotometry. A study of the absorption spectra of nicotine and its analogs was made by Brice and coworkers (*42*). In the ultraviolet region pyridine has an absorption maximum at 257 mμ, which is greatly intensified upon acidification. The absorption spectrum of nicotine is similar to that of pyridine. The slight differences in the curves were expected as a result of substitution of a pyrrolidine nucleus on the pyridine nucleus. As expected also, the absorption curve of nornicotine is similar to that of nicotine. Introduction of a double bond in conjugation with the pyridine ring results in a shift of absorption to longer wave lengths, accompanied by the appearance of a new strong, structureless band at 234 mμ. Presumably, this latter band represents a bathochromic shifting through conjugation of the pyridine absorption normally lying below 200 mμ. Addit of another conjugated double bond, as in nornicotyrine, results in further shifting of the absorption toward the red, with complete loss of the fine structure associated with the pyridine nucleus. Acidification of these compounds leads to further shifts to longer wave lengths, accompanied by an increase in intensity, particularly of the low wave length band. Ultraviolet spectrophotometry cannot distinguish between nicotine and nornicotine nor between various derivatives possessing equivalent degrees of conjugated unsaturation.

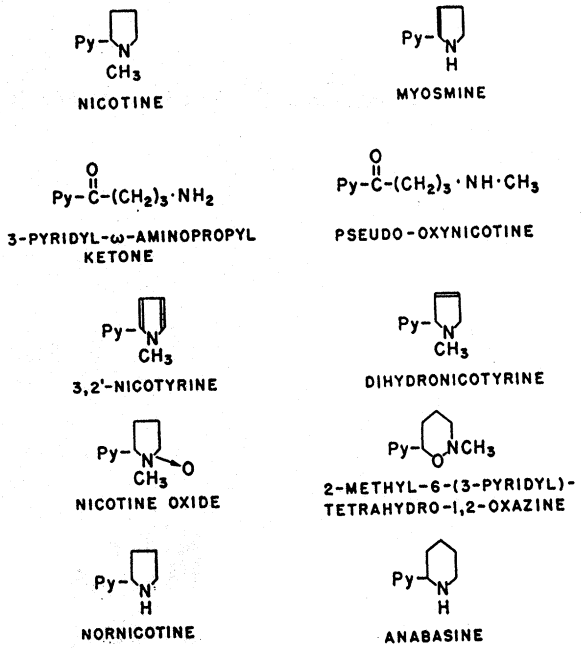

Figure 1. Structural Formulas of Nicotine and Some Related Alkaloids

Py = Pyridine

Paper Chromatography. That the minor alkaloids of tobacco are a complex mixture had been established by many investigators (Pinner, Pictet, Ehrenstein, Späth, and others), who attempted to isolate and identify them. The similarity in properties of these bases makes separation by ordinary physical means extremely difficult. Recently Porter, Naghski, and Eisner (*35*) have shown that paper chromatography is an excellent tool for the resolution of mixtures of alkaloids. By proper choice of solvents, nicotine, nornicotine, anabasine, and nine of their derivatives could be separated by their position on the chromatograph. For further identification, each alkaloid was eluted from the paper, and its spectral absorption curves were determined in the ultraviolet; comparison with curves for pure materials, similarly partitioned and eluted, served to substantiate the identification. Because the optimum concentration of alkaloid for this technique is 10 to 50 mg. per spot of 1-cm. diameter, the method should be applicable not only to the isolation and identification of tobacco alkaloids in the plant, but also for studying the genesis of alkaloids, their fate during fermentation, their metabolism by animal tissues, and their resolution in pyrolytic products such as tobacco smoke.

Analysis of the alkaloids from shed-cured Pennsylvania seed leaf by paper chromatography indicated that, in addition to nicotine and nornicotine, at least three alkaloids are present, bu their chemical identities have not been established. One of these gave R_F values and an ultraviolet absorption curve identical with those of anabasine. Application of this technique to the alkaloids of Maryland tobacco showed that in addition to nicotine

and nornicotine, several other alkaloids were present in minor amounts. The identity of anabasine was confirmed by comparison of x-ray diffraction patterns of the crystalline picrates (*32*). This indicates that anabasine, first reported from a Kentucky-type tobacco by Ehrenstein (*15*), occurs more widely than at first anticipated.

Countercurrent Distribution. Development of countercurrent distribution techniques and apparatus by Craig and coworkers (*6, 7, 49*) has made possible the easy measurement and application of those physical constants which aid in the identification and resolution of unknown substances. Golumbic (*24*) has developed the relationship between partition coefficient and ionization constant as a function of hydrogen ion concentration which is applicable to either mono- or dibases or acids. Thus, by measuring the observed partition coefficient of an acid or base between a solvent and a buffer system as a function of pH, its ionization constants can be calculated. Suitable conditions of pH can then be prescribed for the separation of these materials. If the partition coefficients of the substances are known, it can be predicted in which tubes a preponderance of each substance will fall, as well as the degree of purity attained. Conversely, distributing an unknown mixture will allow calculation of the partition coefficients of the substances and aid in identification. Golumbic (*23*) has applied this technique to various methyl-substituted pyridine and quinoline derivatives, and Badgett (*2*) has applied it to model alkaloids and to nicotine derivatives.

Infrared Spectrophotometry. The infrared portion of the spectrum can be used not only to identify characteristic bonds but also to "fingerprint" an organic molecule. At the shorter wave lengths (2 to 10 microns), each bond has a characteristic vibration, which may be modified by the presence of adjoining groups. Thus, the absorption frequencies in this zone can be used to identify the groups present and to obtain information about the relative positions of these groups in the molecule.

In the region of 3.0 microns, the absorption due to the N—H bond is strong for nornicotine, but for nicotine it is absent. Another point of difference is the presence of considerable fine structure in the nicotine curve in the region of 3.5 microns, which is attributed to the *N*-methyl group.

At longer wave lengths (10 to 15 microns), large sections of the molecule vibrate. Because different molecules have different vibrational frequencies in this region, this portion of the infrared spectrum constitutes a "fingerprint" by which the substances can be identified exactly. Eddy (*14*) has obtained the infrared spectra of nicotyrine, nornicotyrine, metanicotine, dihydrometanicotine, and myosmine.

For purposes of identification, the technique is useful, but of course the substances must be in a relatively pure state. Infrared spectrophotometry can also be used for quantitative determinations, but it is not easily applicable to mixtures of over two components.

Application of much of the information gained from the foregoing studies has resulted in improvement in and even new methods of analysis of the alkaloids.

Quantitative Methods of Analysis

Nicotine. Nicotine is the main alkaloid in tobacco and the one that has been determined most frequently. Methods based on various physicochemical properties have been employed, with various degrees of success. Schloesing (*38*) extracted the tobacco with ammoniacal ether and titrated the free base with standard acid. In 1909 Bertrand and Javillier (*3*) introduced the precipitation of nicotine by silicotungstic acid. In 1911 Chapin (*5*) evaluated a number of methods and concluded that Bertrand's method was on a firm basis. This led to its adoption as an official method by the Association of Official Agricultural Chemists. Although the method yields accurate results with most samples, it is extremely slow, requiring 24 to 48 hours. Moreover, as shown by Ogg *et al.* (*33*), it is subject to serious errors when large amounts of ammonia or ammonium salts are present.

Other investigators—Avens and Pearce (*1*) and Griffith and Jeffrey (*25*)—have attempted to shorten the time for analysis of nicotine in tobacco and similar materials by employing micro and semimicro distillation techniques. A later modification enables 99% of the nicotine to be distilled in 30 minutes.

Recently a spectrophotometric method (*50*) for rapid analysis of nicotine has been developed. The reliability of the method was checked by comparison with the official AOAC silicotungstic acid procedure. The methods were equally reliable at a concentration of 100 mg. of nicotine per 100 ml., but at 10 mg. per 100 ml. the spectrophotometric values were more reliable. Furthermore, the spectrophotometric method showed no interference from $(NH_4)_2SO_4$, Na_2SO_4, or NH_4Cl in concentrations up to 10%; it is very rapid and free of solubility errors inherent with the gravimetric procedure. Thus, the analytical determination of nicotine in tobacco and tobacco products has finally reached a point of refinement where it can be determined both accurately and rapidly.

Wolff *et al.* (*51*) have developed a micromethod for nicotine in blood, based on the color reaction with cyanogen bromide and the finding that Nuchar C absorbs nicotine quantitatively.

Minor Tobacco Alkaloids. Of the minor alkaloids in tobacco, nornicotine has been obtained from an ever-increasing number of types of tobacco. In some it is the predominating alkaloid (in Robinson strain of Maryland and in Cash variety of flue-cured) or is equal to the nicotine in content (some of the burley tobaccos selected genetically for low nicotine content). Attempts to classify tobaccos into nicotine or nornicotine types were made by Markwood and Barthel (*30*) and Bowen and Barthel (*4*) by determination of the melting points of the uncrystallized picrates prepared from the isolated mixture of bases. Later attempts at quantitative estimation of nornicotine took advantage of the fact that it would form a nitroso compound which was not steam volatile. The nicotine was then isolated and determined. In a second aliquot the nornicotine was converted to nicotine by methylation with formaldehyde and formic acid. The difference in the two values was calculated as nornicotine.

Larson and Haag (*29*) proposed a colorimetric procedure for the simultaneous determination of nicotine and nornicotine based on the fact that, under carefully controlled conditions, nornicotine gave a colored compound with cyanogen bromide with an absorption maximum at 540 mμ, whereas that for nicotine was at 375 mμ.

Smith (*39*) found that nicotine formed an azeotropic mixture with steam. This property has been used in separating nicotine from the other alkaloids by distilling a water solution through an efficient fractionating column (*35*). The nornicotine remaining in the residue was determined chemically or spectrophotometrically. If the presence of additional alkaloids was suspected, the nornicotine was methylated to nicotine, and the newly formed base was recovered by distillation.

Feinstein and McCabe (*17*) developed a new quantitative color reaction for nornicotine that is free of interference by nicotine and anabasine. The reagents are acetone, diisopropyl ketone, *p*-hydroxybenzoic acid, and 1,3-diketohydrindene.

They have also developed qualitative tests for nicotine, nornicotine, and anabasine in which they employ Meltzer's reagent and a vanillin-phosphoric acid reagent (*16*).

Reciprocal Grafts with Nicotiana

By developing microprocedures for the tobacco alkaloids and the technique of reciprocal grafts with a non-nicotine-bearing plant, Dawson was able to shed considerable light on the formation, translocation, and possible metabolic significance of the alkaloids. He showed that nicotine was synthesized in the root of *Nicotiana tabacum* (*8, 9, 12*), that anabasine was formed in both

the root and shoot of *N. glauca* but the nicotine formation was restricted to the root (*10*), and that nicotine formed in the roots of *N. glutinosa* was demethylated to nornicotine in the leaves (*11*).

Radioactive Tracers

Radioactive elements are becoming rapidly more important in clarifying the details of the physiology of both animals and plants. Because of the extreme sensitivity of radioactive techniques, tracer elements enable detection of fragments which no longer show a resemblance to the parent compound. Dean *et al.* (*18*) have used P^{32} to study the absorption and utilization of fertilizer phosphorus by tobacco plants. Geiling *et al.* (*22*) synthesized radioactive nicotine by growing *N. rustica* in an atmosphere containing $C^{14}O_2$.

Statistical Mathematics

It has long been known that variations in soil nutrients affect the chemical composition of tobacco leaf. The exact relations, however, were usually hazy, because there are so many nutrients and so many constituents in the leaf. The work of Woltz *et al.* (*52*) is an excellent example of the use of statistics in describing the interplay of the nutrients on the leaf composition. By multiple regressions and prediction equations, they showed that sugar content was inversely related to total nitrogen and directly related to phosphorus and chlorine. Potassium, calcium, and magnesium had no effect. Nicotine content was directly related to nitrogen and calcium and inversely related to phosphorus. Potassium, magnesium, and chlorine had no effect.

Another application of statistics was Wadley's procedure (*45*) for quantitative evaluation of the degree of synergism between two insecticides. This method was used by Mayer *et al.* (*31*) in their successful search for synergists for nicotine.

Statistical treatment of data is rapidly gaining favor, and no doubt there are many phases in the whole tobacco industry where it can be profitably used.

Summary

As an illustration of the value of newer techniques in solving chemical problems in tobacco, recent work on their application to tobacco alkaloids is discussed. It is suggested that what has been so fruitful with alkaloids will be equally fruitful for other groups of tobacco constituents—proteins, nitrogenous compounds other than nicotine, carbohydrates, polyphenols, resins, and volatile substances.

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